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ASSEMBLY OF ACETYLCHOLINESTERASE TETRAMERS BY PEPTIDIC MOTIFS FROM THE MEMBRANE-ANCHOR, PRIMA; COMPETITION BETWEEN DEGRADATION AND SECRETION PATHWAYS OF HETEROMERIC COMPLEXES*

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Running title: Assembly of AChE tetramers by PRiMA

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The membrane-bound form of acetylcholinesterase (AChE) constitutes the major component of this enzyme in the mammalian brain. These molecules are hetero-oligomers, composed of four AChE catalytic subunits of type T ($AChE_T$), associated with a transmembrane protein of type 1, called PRiMA (Proline Rich Membrane Anchor). PRiMA consists of a signal peptide, an extracellular domain that contains a proline-rich motif (14 prolines with an intervening leucine, P_4LP_{10}), a transmembrane domain and a cytoplasmic domain. Expression of $AChE_T$ subunits in transfected COS cells with a truncated PRiMA, without its transmembrane and cytoplasmic domains (P_{stp54} mutant), produced secreted heteromeric complexes (T_4-P_{stp54}), instead of membrane-bound tetramers. In this study, we used a series of deletions and point mutations to analyze the interaction between the extracellular domain of PRiMA and $AChE_T$ subunits. We confirmed the importance of the poly-proline stretches and defined a peptidic motif ($RP_4LP_{10}RL$), which induces the assembly and secretion of a heteromeric complex with four $AChE_T$ subunits, nearly as efficiently as the entire extracellular domain of PRiMA. It is noteworthy that deletion of the N-terminal segment preceding the prolines had little effect. Interestingly, short PRiMA mutants, truncated within the proline-rich motif, reduced both cellular and secreted AChE activity, suggesting that their interaction with $AChE_T$ subunits induces their intracellular degradation.

In the nervous tissue and muscles of mammals, acetylcholinesterase (AChE, EC 3.1.1.7) controls cholinergic transmission by rapidly hydrolyzing the neurotransmitter acetylcholine after its release from nerve terminals. The functional localization of AChE depends on the association of its T splice variant with structural proteins (1-4). Thus, the physiologically active AChE species correspond essentially to the collagen-tailed forms at the neuromuscular junctions and to membrane-bound tetramers in the brain.

The $AChE_T$ splice variant is characterized by its 40-residues C-terminal peptide (t peptide), which contains a C-terminal cysteine and seven aromatic residues, including three evenly spaced tryptophans, and can be organized as an amphiphilic alpha helix (5). This peptide behaves as an autonomous interaction domain (the Tryptophan (W) Amphiphilic Tetramerization domain, or WAT) (6): it allows oligomerization of $AChE_T$ subunits into homomeric dimers (T_2) and tetramers (T_4), as well as heteromeric associations of tetramers with anchoring proteins (7-9).

In the collagen-tailed forms, $AChE_T$ tetramers are associated with a specific collagen, called ColQ (7,10). This interaction has been extensively studied: it is based on a tight interaction between four t peptides (6) and a proline-rich motif, called PRAD (″Proline-Rich Attachment Domain″), located in the N-terminal non

collagenous region of ColQ (11). Synthetic t and PRAD peptides (respectively 40 and 15 residues) spontaneously form a complex, the structure of which has been determined by crystallography: four alpha-helical t peptides form a staggered coiled-coil around the PRAD, organized as an elongated poly-proline II helix (12). All aromatic residues are oriented towards the interior of this compact cylindrical complex, and the tryptophans are apposed to the rings of the proline residues. From this interaction, it was possible to deduce models for the quaternary organization of four $AChE_T$ subunits linked to a ColQ chain (12,13).

The existence of an N-glycosylated 20 kDa hydrophobic protein, associated with membrane-bound AChE tetramers was originally discovered by Gennari and Brodbeck and by Inestrosa *et al*., in 1987 (14,15); this membrane anchor was more recently cloned and called PRiMA ("Proline-Rich Membrane Anchor"), because it contains a proline-rich motif, like ColQ (16). This suggests that $AChE_T$ subunits may associate with PRiMA and with ColQ in a similar manner. However, there are significant differences in the numbers of prolines (8 in ColQ, 14 in PRiMA) and in the numbers and positions of cysteines that could form intercatenary disulfide bonds with a cysteine located near the C-terminus of each of the four t peptides. In addition, PRiMA contains a putative N-glycosylation site between the poly-proline stretches and the transmembrane domain (17). Alternative splicing produces two PRiMA variants, which differ in their C-terminal domains: the intracellular domains of the major variant (PRiMA I) and of the minor variant (PRiMA II) contain respectively 40 and 11 residues (18). The mode of association between PRiMA and $AChE_T$ subunits is physiologically important since the resulting PRiMA-anchored AChE tetramers (16,18) represent the major enzyme species in the brain (18) and their level is regulated by exercise in muscles (19).

We have undertaken an analysis of the association of $AChE_T$ with PRiMA. In this study, we mostly used truncated PRiMA mutants containing only the extracellular domain or fragments of this domain, but not the transmembrane and cytoplasmic domains, thus producing soluble heteromeric complexes with $AChE_T$ subunits. In previous studies, we have shown that a significant fraction of $AChE_T$ subunits is degraded intracellularly, through the ERAD process ("Endoplasmic Reticulum Associated Degradation") (20,21), and that this is mostly induced by exposed aromatic residues (21,22), in agreement with the fact that the formation of a complex in which these residues are occluded may reduce their degradation and increase their secretion. In the present study, we examine how co-expression with PRiMA mutants affects the trafficking, degradation and secretion of $AChE_T$ subunits. We show that some truncated mutants of PRiMA act as degradation inducers, assembling $AChE_T$ subunits into complexes which are degraded intracellularly, indicating that they fail to pass the quality control of the secretory pathway. Using deletions and mutations, we analyze the influence of residues flanking the poly-proline stretches and also of the leucine located between the prolines, and we define a peptidic motif ($RP_4LP_{10}RL$), which is nearly as efficient as the complete extracellular domain of PRiMA for recruitment of $AChE_T$ subunits into secreted heteromeric complexes.

Experimental Procedures

Vectors and site directed mutagenesis - The $AChE_T$ subunit of rat AChE, as well as intact or mutated mouse PRiMA were expressed by inserting the corresponding cDNAs into pEF-Bos vectors (23). Throughout the present article, the numbering of PRiMA residues corresponds to the mature protein (Fig. 1A); the extracellular domain corresponds to residues 1-53. Mutagenesis was performed by the method of Kunkel et *al*. (24), as described previously (25). For deletions, we used mutagenic oligonucleotides of about 30 nt containing 15 nt on each site of the deleted fragment. Truncated mutants are indicated by the position of stop codons and by the modified residues: for example, R36E-P_{stp37} indicates a mutant in which a stop codon was introduced at position 37 and arginine R36 was replaced by a glutamic acid. Fig. 1B shows the structure of mutants used in this study. We introduced a flag epitope (DYKDE) after the cleavage site of the signal peptide in PRiMA, so that it was recognized by the anti-flag monoclonal antibody M1 (Sigma). We also used an N-terminal

fragment of *Torpedo* ColQ, with or without its PRAD domain (Fig. 1C).

Transfection in COS cells - Plasmids were transfected in COS cells with the DEAE-dextran method, as described previously (9), using 2 μg of vector DNA encoding the catalytic subunit $AChE_T$ and various amounts of vector DNA encoding PRiMA mutants, as specified, per 60 mm dish. For comparison, we also used an N-terminal fragment of *Torpedo marmorata* ColQ (Q_{stp69}). In each series of transfections, we completed the amount of vector encoding PRiMA mutants with a vector encoding a non interacting protein, the N-terminal domain of ColQ from which the PRAD interaction motif was deleted (Δ[28-44]-Q_{stp69}) (11), so that the total amount of vector remained constant, to avoid changes in the synthetic capacity of the cells. After transfection, COS cells were incubated at 37°C, in a medium containing 10% NUserum (Inotech, Dottikon, Switzerland), which had been pre-treated with 10^{-5} M soman to inactivate serum cholinesterases. The medium and the cells were collected after 3-4 days.

Analysis of AChE recovery after irreversible inhibition - The AChE activity of transfected cells (3 or 4 days after transfection) was irreversibly inhibited by incubation with the membrane-permeant inhibitor soman (pinacolylmethyl-phosphonofluoridate) at 5 10^{-7} M for 30 min, as described in (22). After extensive washing, the recovery of AChE activity was determined by collecting cells at various times in fresh culture medium at 37 °C. Secretion of newly synthesized AChE only resumed after about 150-180 min and during that period the recovery of cellular activity reflected a balance between neosynthesis and intracellular degradation.

Cell extracts - Intracellular and membrane-bound AChE was extracted for 15 min at 20°C in a TMg buffer (1% Triton X-100, 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$) containing 25 mM benzamidine, followed by centrifugation for 10 min at 13 000 r.p.m at 4°C. The culture medium containing the secreted enzyme was also centrifuged at 13 000 r.p.m for 10 min to remove cell debris before analysis.

Enzyme assays - AChE activity was determined with the colorimetric method of Ellman *et al.* (26) at room temperature. The reaction was monitored at 414 nm with a Labsystems Multiskan RC automatic plate reader (Helsinki, Finland); the optical density was recorded at 20 s intervals over a period of 10 min. Alkaline phosphatase and β-galactosidase from *Escherichia coli* were assayed with the chromogenic substrates p-nitrophenyl phosphate and o-nitrophenyl galactoside, respectively.

Sedimentation and electrophoresis analyses - Centrifugation in 5-20 % sucrose gradients (50 mM Tris-HCl pH 7.5, 20 mM $MgCl_2$, in the presence of 1 % Brij-96 or 0.2% Triton X-100) was performed in a Beckman SW41 rotor, at 36 000 r.p.m, for 17 h 30 at 6°C. Approximately 40 fractions were collected and AChE activity was measured with the Ellman colorimetric assay, allowing the determination of the sedimentation coefficients of the different molecular forms, and of their relative activities. The gradients contained *E. coli* β-galactosidase (16 S) and alkaline phosphatase (6.1 S) as internal sedimentation standards. Amphiphilic molecules are characterized by the fact that they interact with detergent micelles: they generally sediment slower in the presence of Brij-96 than of Triton X-100.

Electrophoresis in non-denaturing polyacrylamide gels was performed as described by Bon *et al.* (27); The gels contained 0.25% Triton X-100 with or without 0.05% deoxycholate; electrophoresis was performed in a refrigerated apparatus under 40 V/cm, for 2 h. Enzymatic activity was revealed by the histochemical method of Karnovsky and Roots (28). This method allows a rapid qualitative comparison of up to 20 samples in a single gel. In charge shift electrophoresis, the electrophoretic migration of amphiphilic molecules was accelerated in the presence of sodium deoxycholate, when compared to migration in the presence of the neutral detergent, Triton X-100.

Effect of synthetic peptides on oligomerization of $AChE_T$ subunits - Synthetic peptides corresponding to the interaction motif of PRiMA, (RP_4LP_{10} and $RP_4LP_{10}RL$) were synthesized by the Merrifield solid phase method in an Applied Biosystems (ABI) 431A automated peptide synthesizer, with small-scale 9-fluorenylmethoxycarbonyl (Fmoc)

chemistry (29). Protected amino acids were purchased from Applied Biosystems (Foster City, CA) (30). Crude peptides were purified by reverse-phase HPLC on a Vydac C18 column (5 µm, 250 x 10 mm^2), using appropriate acetonitrile gradients containing 0.1% TFA.

A 10^{-2} M solution of peptide in Tris-HCl pH 8 (1 M) was added with fresh medium to transfected COS cells expressing only $AChE_T$ subunits, at a final concentration of 10^{-4} M. Peptides were also added to cell homogenates, at a final concentration of 10^{-4} M, and incubated overnight at 20°C or 37°C. In some samples, a synthetic t peptide (WAT) was added at 2 10^{-4} M. The oligomeric state of AChE was then analyzed by non-denaturing electrophoresis, after dilution two-fold in buffer containing 1% Triton X-100.

RESULTS

PRiMA mutants - Fig. 1A shows the primary sequence of mouse PRiMA, and the structure of mutants used in this study. PRiMA comprises a signal peptide (residues -35 to -1), an extracellular N-terminal domain (residues 1 to 53) that includes four cysteines, a proline-rich motif and a putative N-glycosylation site, a transmembrane domain (residues 54 to 78) and two possible cytoplasmic C-terminal domains, that define PRiMA I and PRiMA II. We studied the interaction of $AChE_T$ subunits with the extracellular domain of PRiMA, using mutants lacking the transmembrane and cytoplasmic domains (Fig. 1B). Firstly, we introduced stop codons at various positions (n), producing proteins P_{stpn}. Secondly, we deleted most of the N-terminal region preceding the prolines, in mutants designed as Δ_N; to maintain a correct signal peptide cleavage site, we conserved the first or the last residue of this region (E1 and R20, respectively). We also assessed the possible influence of electrostatic effects, by mutating the residue preceding the prolines by other charged or uncharged residues (D, K or A). Finally, we analyzed the role of the leucine located within the poly-proline region (L25), by replacing it with a proline, and by adding a leucine at position 31, with or without the original leucine. Thus the original motif P_4LP_{10} was changed to P_{15}, $P_{10}LP_4$ and $P_4LP_5LP_4$.

Formation and secretion of soluble complexes with truncated PRiMA mutants, lacking the transmembrane domain - PRiMA and its different mutants were expressed in COS cells together with $AChE_T$ subunits (also called T subunits). To evaluate the formation of heteromeric complexes, we analyzed the total AChE activity of the cellular extracts and of the medium, as well as their composition in molecular forms, using both sedimentation and non-denaturing electrophoresis. It should be noted that all molecular forms of AChE, which differ in their degree of oligomerization, with or without associated proteins, possess the same catalytic activity per active site, so that the level of AChE activity reflects the number of $AChE_T$ subunits, independently of the composition in molecular forms.

Fig. 2 illustrates the molecular forms obtained by expressing $AChE_T$ subunits alone, with full-length PRiMA and with its extracellular fragment, P_{stp54}. As a control, we used an N-terminal fragment of ColQ which contains the PRAD (residues 28-44), without the collagenous and trimerization domains (Q_{stp69}) (Fig. 1C). When expressed alone or in the presence of a non interacting fragment of ColQ from which the proline-rich motif was deleted (Δ[28-44]-Q_{stp69}), T subunits produced mostly amphiphilic monomers (T_1) and dimers (T_2) (27), with a small proportion of nonamphiphilic tetramers sedimenting at 10.5 S, which are thought to be homomeric (T_4), i.e. with no associated endogenous non catalytic component (Fig. 2A). Monomers and dimers are not readily resolved in the sedimentation profiles, and we therefore quantified their sum (T_1+T_2). The proportion of T_4 tetramers is markedly higher in the medium than in the cell extract, indicating that they are secreted more efficiently than monomers and dimers, as previously observed (9).

Co-expression of T subunits with full length PRiMA induced the formation of membrane-bound T_4-PRiMA complexes, expressed at the cell surface as demonstrated by immunofluorescence (not shown). These heteromeric complexes were recovered in the cell extract after solubilization in the presence of 1% Triton X-100; unlike homomeric tetramers (T4), which sediment at 10.5 S regardless of the presence or absence of detergents, the PRiMA-associated tetramers are

amphiphilic and their sedimentation is influenced by detergents: they sediment at 9.8 S in the presence of Triton X-100 and 9 S in the presence of Brij-96. This was also shown by the fact that they migrated more slowly than T_4 tetramers in non-denaturing electrophoresis (Fig. 2B). In addition, the medium contained an increased level of soluble molecules sedimenting at 10.5 S, suggesting that they correspond to nonamphiphilic tetramers. When $AChE_T$ subunits were co-expressed with an N-flagged PRiMA, these secreted tetrameric molecules were recognized by an anti-flag antibody (M1), as shown by retardation of their migration in non denaturing electrophoresis (Fig. 3), demonstrating that they contain an N-terminal fragment of PRiMA, that might be produced either intracellularly or at the cell surface.

Co-expression of $AChE_T$ subunits with the P_{stp54} mutant, corresponding to the extracellular domain of PRiMA, induced the formation of a soluble AChE form, sedimenting at 10.5 S, and migrating as an AChE tetramer in non-denaturing electrophoresis (Fig. 2A and B). This form, which could not be distinguished from homomeric T_4 tetramers by sedimentation, corresponds to $AChE_T$ tetramers associated with the P_{stp54} protein (T_4-P_{stp54}). The fact that the ratio of T_4-P_{stp54} to (T_1+T_2) is higher in the medium than in the cell extract, shows that they are preferentially secreted, as observed for homomeric tetramers T_4.

The formation of soluble T_4-P_{stp54} complexes demonstrates that the amphiphilic character of T_4-PRiMA complexes is due to the transmembrane domain of PRiMA and confirms that $AChE_T$ subunits interact with a peptidic motif located in the N-terminal extracellular region of PRiMA, in agreement with a previous study (16).

Effect of progressive C-terminal deletions in the extracellular domain of PRiMA on the recruitment of $AChE_T$ subunits - We examined the interaction between $AChE_T$ subunits and PRiMA mutants in which stop codons were introduced upstream of position 54 in mutants P_{stp46}, P_{stp43}, P_{stp39}, P_{stp38}, P_{stp37}, P_{stp36}, P_{stp33} and P_{stp31} (see Fig. 1B). We studied the AChE activity and molecular forms produced in COS cells expressing a constant amount of $AChE_T$ subunits (2 µg of vector DNA per 60 mm culture dish), together with varying amounts of each mutant.

The proportion of $AChE_T$ tetramers increased in the cells and in the medium with the amount of associated non catalytic protein, as illustrated for P_{stp33} and P_{stp54} and Q_{stp69} in Fig. 4. This proportion was found to plateau at similar values for P_{stp54} and Q_{stp69}, but at a much lower level for P_{stp33}.

Fig. 5 illustrates the effect of the P_{stpn} mutants on the cellular activity and on the rate of secretion, with a fixed amount of plasmid encoding the P_{stpn} mutants (2 µg of DNA encoding $AChE_T$ and PRiMA mutant, each, per 60 mm culture dish). The level of cellular activity varied very little when $AChE_T$ subunits were co-transfected with the various PRiMA mutants. Secretion increased by about 30% when $AChE_T$ subunits were co-transfected with Q_{stp69}, indicating that they were partially rescued from intracellular degradation by the formation of T_4-Q_{stp69} complexes (not shown), but reduced by about 50% when they were co-transfected with P_{stp31}. The secreted activity gradually increased with the longer mutants P_{stp33} and P_{stp36}, approximately reaching the value obtained with $AChE_T$ alone for longer constructs.

The level of secreted (T_1+T_2) was markedly reduced, when $AChE_T$ subunits were co-transfected with any of the truncated P_{stpn} mutants, as well as with Q_{stp69}, indicating that they interacted with all mutants, even the shorter ones, such as P_{stp33} or P_{stp31}, which produced only minimal levels of cellular or secreted T_4-P_{stpn} form. The variations observed in the level of secreted activity appeared correlated with the proportion of heteromeric complex in the medium, as shown in Fig. 5C. Remarkably, this correlation includes all the truncated PRiMA mutants analyzed in this study, and also Q_{stp69}. Fig. 5D shows that the secretion of T_4-P_{stpn} complexes appears proportional to their cellular activity, except for T_4-P_{stp36}, which was more efficiently secreted, and for mutants containing A, D or E residues preceding the proline-rich segment, which are less efficiently secreted.

The fact that AChE activity was reduced, both in the cells and in the medium, when $AChE_T$ subunits were co-expressed with short truncated

PRiMA mutants such as P_{stp31} suggests that they were more degraded than when expressed alone. This was verified by following the initial rate of recovery of AChE activity, during 2 hours after irreversible inhibition, i.e. before secretion of the newly synthesized enzyme: this rate represents the balance between neosynthesis and intracellular degradation. The rate of neosynthesis must be identical when $AChE_T$ subunits are expressed with a non interacting protein and with different PRiMA mutants. We found that the rates of recovery varied in the order $AChE_T + \Delta[28\text{-}44]\text{-}Q_{stp69} > AChE_T + P_{stp46} > AChE_T + P_{stp33}$, indicating that P_{stp33}, and to a lesser degree P_{stp46}, induced some degradation of newly synthesized active $AChE_T$ subunits.

The ratio of secreted to the cellular activity of each molecular form of AChE can be considered as an index of its secretability: Fig. 6 shows that this ratio remained essentially constant for (T_1+T_2), which were the same in all cases, but varied markedly for AChE tetramers associated with P_{stpn} proteins. This indicates that the secretion of the complexes depended on the length of the PRiMA fragment, especially between P_{stp31} and P_{stp36}, confirming that complexes formed with the shorter PRiMA mutants were not efficiently secreted. In addition, the fact that this ratio varied in a non monotonic manner as a function of the length of the mutants from 36 to 54, suggests that the C-terminal residues of the P_{stpn} proteins may either facilitate or reduce secretion.

The P_{stp38} mutant seemed to produce a maximum of secreted heteromeric complexes, while P_{stp36} appeared optimal for the secretability of such complexes. The differences observed between P_{stp36}, P_{stp37}, P_{stp38} and P_{stp39} show that the residues located immediately downstream of the prolines (RLL) influence the formation and the secretion of the T_4-P_{stpn} complexes. However, replacement of R by an alanine or a glutamic acid in mutant P_{stp37} (R36A-P_{stp37} and R36E-P_{stp37}), or replacement of RLL by alanines in mutant (RLL/AAA-P_{stp39}) had essentially no effect on recruitment of $AChE_T$ subunits, indicating that the charge of these residues is not important.

It is interesting that P_{stp46} and P_{stp54} produced very similar results, because P_{stp54} possesses a putative N-glycosylation site and a cysteine which are removed in P_{stp46}, suggesting that these two elements do not interfere, positively or negatively, with the interaction between PRiMA and $AChE_T$ subunits in transfected COS cells. The lack of influence of these elements was confirmed by mutating cysteine C48 to a serine in P_{stp54}, and by suppressing the glycosylation site by replacing the threonine T46 by an alanine: these mutations did not affect the production and secretion of T_4-P_{stpn} complexes (not shown).

Influence of the region preceding the poly-proline stretches - To assess the possible role of the peptidic region that precedes the poly-proline stretches of PRiMA, we deleted it in P_{stp46} and P_{stp54}. To maintain a proper cleavage site between the signal peptide and the mature protein, the prolines stretches were preceded by an arginine R (as in the wild type), or by other charged or neutral residues (K, E, D, A), as shown in Fig. 1B.

With an arginine preceding the prolines, we found that deletion of the N-terminal fragment (residues 1-19) from mutants P_{stp46} or P_{stp54} (Δ_N-R-P_{stp46} and Δ_N-R-P_{stp46}) slightly reduced the amount of cellular and secreted complexes (Fig. 5B). The fact that the two deleted mutants produced the same effect confirms that residues 45-53 do not influence the assembly or the trafficking of the complexes.

We then examined a possible effect of a charged or neutral residue preceding the prolines, in mutants Δ_N-R-P_{stp46}, Δ_N-K-P_{stp46}, Δ_N-A-P_{stp46}, Δ_N-D-P_{stp46}, and Δ_N-E-P_{stp46} (Fig. 5B and Fig. 6). We found that the amount of secreted AChE tetramers is higher with a neutral or acidic residue, but that their secretability appears lower, as indicated by the ratio of secreted to cellular activities, suggesting that this residue influences the efficiency of secretion (Fig. 5D).

It was surprising that the secretion of AChE complexes was lower with a positively charged residue upstream of the prolines, since this residue is an arginine (R) in the wild type PRiMA. However, when we replaced this arginine by a glutamic acid (E) in P_{stp46}, we found no modification of the yield of T_4-P_{stp46} complexes, indicating that this charge effect is negligible when the N-terminal segment (1-19) is present (not shown).

Mutations in the proline-rich region - PRiMA contains two groups of successive prolines, separated by a leucine residue (P_4LP_{10}). To determine whether this leucine is required for the interaction with $AChE_T$ subunits, we mutated it to a proline, starting from the Δ_N-E-P_{stp46} mutant. The production and secretion of AChE tetramers remained unchanged with the resulting mutant, noted Δ_N-(P_{15})-P_{stp46}. However, the production of complexes was significantly reduced by displacement of the leucine to a symmetrical position in the proline stretches ($P_{10}LP_4$) and even more by addition of a second leucine ($P_4LP_5LP_4$), as shown in Fig. 5B. These mutations had little effect on the secretability of the complexes (Fig. 6). Thus, the recruitment of $AChE_T$ subunits into heteromeric T_4-P_{stp46} complexes is weakened by an interruption of the proline stretches by a leucine, and this suggests that a succession of 10 prolines is sufficient to ensure a maximal efficiency.

A proline-rich peptidic motif, sufficient for recruitment of $AChE_T$ subunits - In the preceding sections, we showed that it is possible to maintain an efficient recruitment of $AChE_T$ subunits into T_4-P_{stpn} hetero-oligomers after deletion of the N-terminal region of PRiMA preceding R20 and of the C-terminal region following L37. Therefore, the 20-37 peptidic motif, which contains the poly-proline stretches ($RP_4LP_{10}RL$), may be sufficient for this interaction. To verify this conclusion, we combined N-terminal and C-terminal deletions in the Δ_N-R-P_{stp38} mutant. This mutant was co-expressed with $AChE_T$ subunits, and compared with P_{stp54}, Δ_N-R-P_{stp38} and P_{stp38}, all expressed at 2 µg vector/dish (Fig. 5). All mutants induced the production of T_4-P_{stpn}, confirming the effects observed with separate deletions of the N-terminal and C-terminal regions. Thus, the 18-residue peptide 20-37, derived from PRiMA, possesses the capacity to assemble four $AChE_T$ subunits into stable T_4-P_{stpn} complexes in a manner similar to that of the full length extracellular domain of PRiMA (P_{stp54}).

It is noteworthy that deletion of the N-terminal region, in Δ_N-R-P_{stp54} and Δ_N-R-P_{stp38} increased the ratio of secreted to cellular T_4-P_{stpn} by about 25%, compared to the corresponding mutants P_{stp54} and P_{stp38}, suggesting that secretion was facilitated by the N-terminal deletion. Deletion of the C-terminal region, in P_{stp38} and Δ_N-R-P_{stp38}, compared to P_{stp54} and Δ_N-R-P_{stp54}, also appeared to increase its secretability.

Assembly of $AChE_T$ tetramers by synthetic peptides - We synthesized the PRiMA-derived peptides RP_4LP_{10} and $RP_4LP_{10}RL$, to determine whether they could induce the assembly of $AChE_T$ monomers and dimers into tetramers. In a first series of experiments, the peptides were added at a final concentration of 10^{-4} M to COS cells expressing only $AChE_T$ subunits, during the transfection process and/or in the culture medium. The cells and the medium were analyzed by non denaturing electrophoresis after 3 days, as indicated above. This showed a clear increase in the proportion of $AChE_T$ tetramers, most probably associated with the peptides (Fig. 7A). This result indicates that the peptides were able to penetrate the biosynthetic compartments of the living cells.

In a second series of experiments, homogenates of COS cells expressing only $AChE_T$ subunits were prepared without detergent, and incubated overnight at 37°C with the peptides (10^{-4} M). As shown in Fig. 7B, both peptides induced the assembly of tetramers, under these acellular conditions. When a synthetic t peptide (WAT), at 2.10^{-4} M, was added to the cell homogenate together with the PRiMA-derived peptides, it reduced markedly the recruitment of $AChE_T$ monomers and dimers into tetramers, indicating a direct interaction between these peptides.

DISCUSSION

The N-terminal extracellular domain of PRiMA assembles $AChE_T$ subunits into soluble T_4-P_{stpn} complexes - The assembly of $AChE_T$ tetramers with the transmembrane protein PRiMA produces membrane-bound complexes, both in the nervous system (14-18,31) and in muscles (19). In addition, the availability of PRiMA seems to limit the stabilization, as well as the functional localization of AChE in the mammalian brain (18). It is therefore important to analyze the interaction between PRiMA and $AChE_T$ subunits. In this

study, we attempted to define the peptidic motif of PRiMA which is responsible for its quaternary association with $AChE_T$ subunits, by using deletions upstream, downstream and within the proline-rich segment.

We mostly used truncated PRiMA mutants, lacking the transmembrane and cytoplasmic domains (P_{stpn} with a stop codon at position n), which were co-expressed with $AChE_T$ subunits in transiently transfected COS cells, producing soluble complexes (T_4-P_{stpn}), which were secreted instead of remaining attached to the cell membrane.

Truncated PRiMA mutants recruit $AChE_T$ subunits into heteromeric complexes, following either secretion or degradation pathways - We have shown in a previous study that a significant fraction of $AChE_T$ subunits is degraded intracellularly through the ERAD process, while another fraction is secreted (20,21). Therefore, the level of intracellular AChE activity reflects a balance between the rate of synthesis and the combined rates of degradation and secretion.

When COS cells were transfected with a fixed amount of plasmid encoding $AChE_T$ subunits, together with various amounts of plasmid encoding PRiMA mutants, a variable fraction of the subunits was integrated in T_4-P_{stpn} complexes, both in the cells and in the medium. It was surprising to find that, although the proportion of T_4-P_{stpn} complexes varied widely, it appeared to plateau for similar amounts of plasmid DNA, suggesting that all mutants presented a similar apparent "affinity" for $AChE_T$ subunits. This paradox can be resolved by assuming that even when the observed proportion of complexes was very low, mutants such as P_{stp31} did in fact interact with $AChE_T$ subunits, as indicated by a marked reduction in the secretion of (T_1+T_2), but produced rapidly degraded complexes. With longer mutants, the level of secreted T_4-P_{stpn} complex progressively increased. Thus, the variations observed in the amount of secreted complex appear to reflect a competition between secretion and degradation. Depending on its organization, each type of T_4-P_{stpn} complex would be oriented with different probabilities towards the secretion pathway or the degradation pathway. This suggests that the rate of secretion depends critically on the structure of the complex.

It is a classical notion that proteins destined to function as a multi-subunit complex may be retained in the endoplasmic reticulum by the quality control system of the cell, unless they are appropriately associated with their partners (32). This can be rationalized by the fact that they expose hydrophobic surfaces in their unassembled state, but not in the complex. This seems to be the case for *Torpedo* $AChE_T$ subunits, which are very little secreted when expressed alone in COS cells, but can be secreted when co-expressed with an N-terminal fragment of ColQ (Q_{stp69}) (5). We also observed here that rat $AChE_T$ subunits can be rescued by Q_{stp69} from degradation through ERAD (20). This is consistent with the fact that their physiological function depends on association with an anchoring protein, ColQ or PRiMA.

The fact that short truncated PRiMA mutants increase the intracellular degradation of $AChE_T$ subunits is more unusual. However, Ulloa-Aguirre and Conn recently suggested that some proteins induce an inappropriate conformation of normally externalized molecules and thus target them towards degradation by the cellular quality control, and proposed to call this hypothetical process "protein shipwrecking" (33). Here, we present evidence that some PRiMA mutants may behave in this manner, and that there is in fact a continuous spectrum between mutants which induce degradation and those which rescue $AChE_T$ subunits from ERAD and promote their secretion as heteromeric complexes.

Since the assembly of a complex between proteins occludes interaction surfaces, rather than exposing hydrophobic zones, how could the association with short PRiMA mutants such as P_{stp31} induce the degradation of $AChE_T$ subunits? Although we have presently no experimental evidence, we may propose an hypothetical explanation. We have previously demonstrated that aromatic (but not hydrophobic) residues of the C-terminal t peptide of AChE play a crucial role to induce the degradation of $AChE_T$ subunits by ERAD (22). It is possible that a group of aromatic residues remains exposed in the complexes formed with short PRiMA mutants, engaging their translocation into the cytoplasm and degradation by proteasomes, whereas these residues are masked in

complexes formed with longer mutants, as demonstrated by crystallography in a complex of four t peptides with the PRAD of ColQ (12).

Definition of a sufficient interaction motif (PRAD) in PRiMA - The efficiency of secretion of AChE oligomers increases with their degree of oligomerization (9). The secretability of heteromeric T_4-P_{stpn} complexes, indicated by the ratio of secreted to cellular activity, was variable: it is noteworthy that it was increased by deletion of peptidic regions located upstream or downstream of the poly-proline stretches. A maximum was obtained by removing all residues following the prolines (P_{stp36}); conversely, it was reduced for mutants in which the N-terminal region was deleted (Δ_N) and the proline-rich segment was preceded by residues A, E, or D, as opposed to basic residues R or K.

Most of the N-terminal and C-terminal regions flanking the poly-proline stretches could be deleted without suppressing the capacity of P mutants to engage $AChE_T$ subunits into T_4-P_{stpn} complexes. This capacity in fact appeared maximal when most of the C-terminal region was deleted, leaving only two residues (RL) after the prolines, in P_{stp73}. However, it is not certain that these residues play a specific role in the association with t peptides, because replacement of the three residues following the prolines by alanines had essentially no effect (in RLL/AAA-P_{stp39} versus P_{stp39}). Even though we have no direct evidence that P_{stp54} is N-glycosylated under our experimental conditions, we found that the presence of a putative N-glycosylation site, located at position 44, did not influence the association of PRiMA mutants with $AChE_T$ subunits. Similarly, the cysteine located at position 48 did not affect the formation of T_4-P_{stpn} complexes.

The PRAD of ColQ is normally disulfide-linked to two of the t peptides, while the other two are disulfide-linked together (5), and it has been reported that, as in the case of ColQ, two of the $AChE_T$ subunits are disulfide-linked with the membrane anchor, in brain AChE (14,15). However, we previously found that the formation of heteromeric complexes with the N-terminal region of ColQ could occur without the two adjacent cysteines preceding the PRAD, indicating that disulfide bonds between ColQ and two $AChE_T$ subunits are not required (5,11). The present results suggest a similar conclusion, since the formation of complexes was only slightly reduced (20% or less) by deletion of most of the N-terminal region, which contains four cysteines, located at positions 6, 13, 17, 19. This does not rule out the possibility that some of these cysteines may be involved in intercatenary disulfide bonds between PRiMA and $AChE_T$ subunits, as shown in membrane-anchored AChE tetramers from bovine brain (14,15,34), and thus stabilize the complex.

PRiMA contains two poly-proline stretches, separated by a leucine (P_4LP_{10}). We found that this leucine is not required for the interaction, and could be replaced by a proline, resulting in a single suite of 15 prolines (P_{15}). The secretion of T_4-P_{stpn} complexes was decreased by displacement of the leucine ($P_{10}LP_4$) or interruption of the proline stretches by a second leucine ($P_4LP_5LP_4$) and it was almost totally abolished when 5 prolines were deleted in P_{stp31} (P_4LP_5). Thus, the formation of secretable complexes requires the presence of a minimal number of uninterrupted prolines.

Our deletion experiments converged on the idea that the 20-37 segment of PRiMA ($RP_4LP_{10}RL$) may be able to recruit $AChE_T$ subunits nearly as efficiently as the entire extracellular domain of PRiMA, and this was confirmed by expressing this 18-residue peptide, as mutant Δ_N-R-P_{stp38}. In fact, addition of the synthetic peptides RP_4LP_{10} and $RP_4LP_{10}RL$ to COS cells expressing only $AChE_T$ subunits induced the recruitment of monomers and dimers into stable tetramers. Moreover, we obtained the same result by incubating a cell homogenate with the peptides, and observed that the assembly of $AChE_T$ tetramers was partially blocked by addition of a synthetic t peptide (WAT). This proves that the PRiMA-derived and t peptides spontaneously interact *in vitro*, as shown previously for a ColQ-derived PRAD peptide (12), and opens the way to the formation, crystallization and structural analysis of the core complex which forms the basis of the association between PRiMA and $AChE_T$. It is noteworthy that the PRAD of ColQ is very efficient with only 8 prolines (P_3MFP_5). This raises the intriguing possibility that the complexes of t peptides with the proline-rich motifs of ColQ and

PRiMA might significantly differ in their quaternary organization. Using short PRiMA-derived peptides, it may also be possible to shed some light on the structural differences between complexes that pass or fail the quality control in the secretory pathway.

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FOOTNOTES

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[1]The abbreviations used are: AChE: acetylcholinesterase (E.C. 3.1.1.7); $AChE_T$: splice variant T; PRiMA: Proline-Rich Membrane Anchor; ColQ: collagen Q; ERAD: Endoplasmic Reticulum Associated degradation; PRAD: Proline-Rich Attachment Domain; P_{stpn}: truncated PRiMA mutant, terminating with a stop codon at position n; Δ_N-X-P_{stpn}: PRiMA mutant, with a deletion of the N-terminal fragment, starting with residue X before the prolines, and terminating with a stop codon at position n; WAT: Tryptophan (W) Amphiphilic Tetramerization domain.

FIGURE LEGENDS

Fig. 1. Structure of PRiMA and of mutants used in this study. **A.** Sequence of PRiMA, numbered from the first residue of the mature protein. The putative signal peptide is shown in *thin letters* (-35 to –1), the extracellular domain in *bold letters* (1 to 53), the transmembrane domain in *bold italics*, and the cytoplasmic domain in *thin italics* (the sequence shown corresponds to the major splice variant PRiMA I, possessing the longer cytoplasmic domain). In the extracellular domain, the putative N-glycosylation site is indicated by an asterisk and the cysteines are underlined. **B.** Structure of mutants. All mutants possess the signal peptide (not shown), and fragments of the extracellular domain, but not the transmembrane and cytoplasmic domains. They are designated by the position of introduced stop codons (e.g. P_{stp54} contains the entire extracellular domain, 1-53). The deletion of the region preceding the poly-proline stretches is indicated by D; in these mutants, a single residue precedes the prolines, so as to maintain a proper cleavage site after the signal peptide: this residue is indicated, e.g. arginine (R) in Δ_N-R-P_{stp54}. Mutants in the proline-rich region are indicated by the distribution of prolines and leucine residues (e.g. P_4LP_{10} in the case of the wild type). The interaction motif ($RP_4LP_{10}RL$) corresponds to mutant Δ_N-R-P_{stp38}. **C.** Structure of the N-terminal fragment of *Torpedo* ColQ (Q_{stp69}) (the signal peptide is not shown). The underlined fragment corresponds to the PRAD interaction domain. It was deleted in Δ[28-44]Q_{stp69}.

Fig. 2. Molecular forms of AChE produced and secreted by transient transfection of COS cells with $AChE_T$ expressed alone, with full length PRiMA and with truncated PRiMA, reduced to its extracellular domain (P_{stp54}). **A.** Representative sedimentation profiles, obtained from gradients containing 1% Brij-96, normalized to the same total AChE activity, indicating the relative proportions of monomers (T_1), dimers (T_2) and tetramers, associated or not with PRiMA or P_{stp54}. Profiles corresponding to cell extracts are shown in the top panel, and to the medium in the middle panel. (—o—): $AChE_T$ expressed alone; (—■—):$AChE_T$ expressed with PRiMA; (- -▲- -): $AChE_T$ expressed with P_{stp54}. In general, the relative proportion in the medium, compared to cell extracts, increases with the degree of oligomerization, indicating that secretion is facilitated by

oligomerization. Soluble non amphiphilic tetramers (T_4) and heteromeric complexes (T_4-P_{stp54}) sediment around 10.5 S, while amphiphilic T_4-PRiMA complexes, containing the transmembrane domain of PRiMA, are retarded by their interaction with the detergent (Brij-96) and sediment around 9 S. **B.** Migration of AChE forms from cell extracts and culture media in non denaturing electrophoresis. In the profiles corresponding to co-transfected cells ($AChE_T$ with PRiMA and $AChE_T$ with the extracellular domain of PRiMA, P_{stp54}), the simple arrows indicate non amphiphilic AChE tetrameric forms; the arrow with an asterisk indicates the amphiphilic tetramer associated with a complete PRiMA protein.

Fig. 3. Secreted AChE tetramers induced by co-transfection of $AChE_T$ subunits with PRiMA contain an N-terminal fragment of PRiMA. The electrophoretic migration of AChE tetramers secreted by COS cells expressing $AChE_T$ subunits with an N-flag-PRiMA was retarded, in non-denaturing gels, after incubation with the monoclonal anti-flag antibody M1. AChE tetramers are indicated by arrows and the retarded component by an asterisk.

Fig. 4. Variation of the proportion of AChE tetramers with the amount of PRiMA mutants. COS cells were transfected with a fixed amount of DNA encoding $AChE_T$ subunits (2 µg per 60 mm culture dish) and the indicated amount of DNA encoding PRiMA mutants, or the N-terminal region of ColQ (Q_{stp69}), complemented with DNA encoding a non interacting protein, so that the total amount of vector was kept constant to avoid modifications in the protein synthesis capacity of the cells. The proportions tetramers, associated or not with P_{stpn} proteins (T_4 + T_4-P_{stpn}) was determined from sedimentation profiles, as in Fig. 2. The figure illustrates the results obtained for P_{stp31} and P_{stp54} mutants, producing respectively high or low proportions of T_4-P_{stpn} complexes. The curves obtained with all other mutants were very similar in shape, differing mostly by the maximal levels of tetramers: co-transfection with 2 µg DNA/dish encoding the various mutants was used in most subsequent experiments.

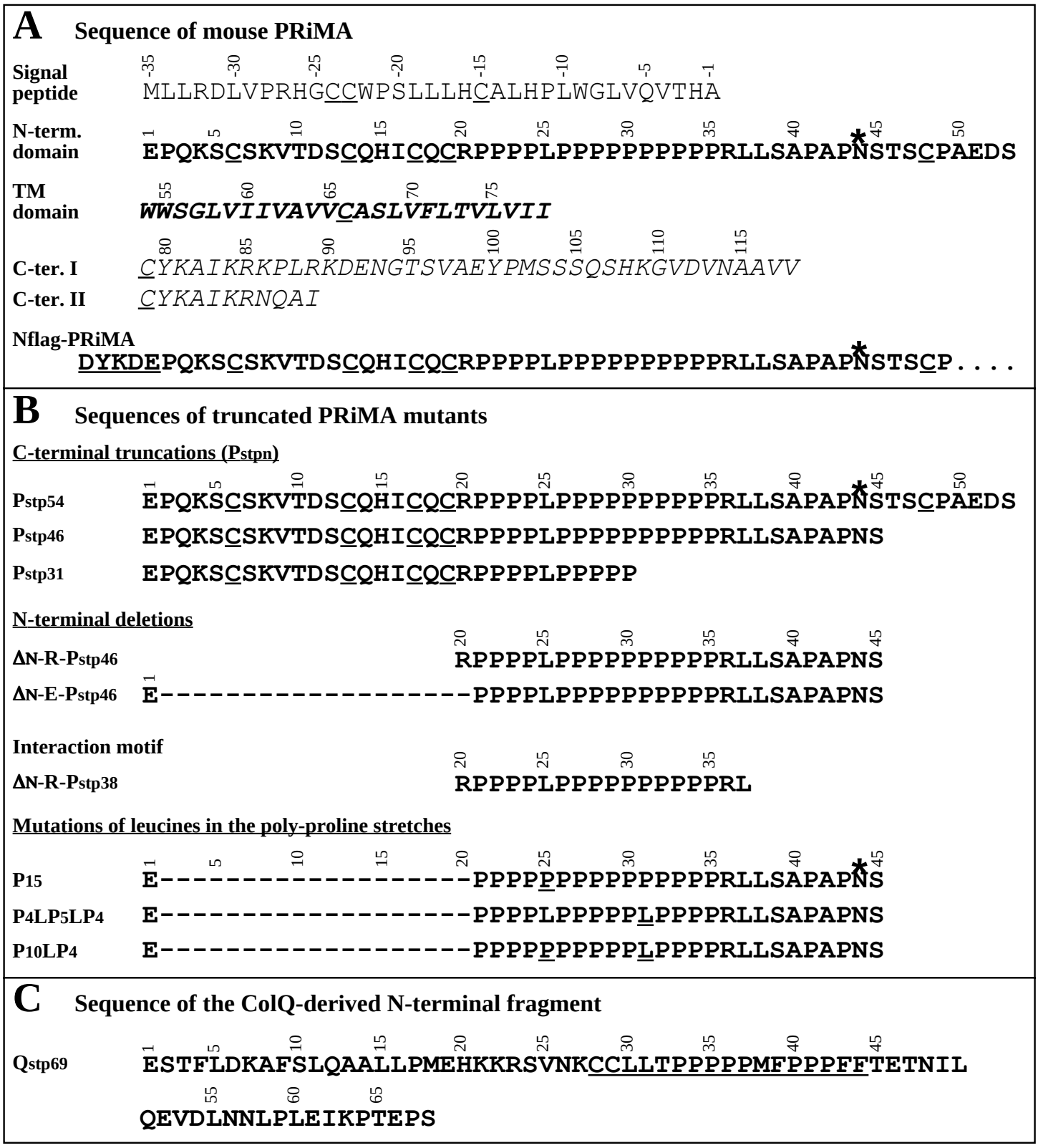
Fig. 5. Effect of the PRiMA mutants on cellular and secreted AChE activity and molecular forms. COS cells were co-transfected with 2 µg of DNA encoding $AChE_T$ and 2 µg of DNA encoding PRiMA mutants. A. Sequence and numbering of the extracellular domain of PRiMA; the cysteines are underlined and the putative N-glycosylation site is indicated by an asterisk. **B.** The cellular and secreted AChE activities corresponding to the different mutants were normalized, for each experiment, to those of $AChE_T$ subunits expressed alone, taken as 100%. The proportions of the molecular forms were determined from sedimentation profiles. The data corresponding to C-terminal truncated mutants (1 to 9) are the means of 4 to 7 independent transfection experiments, ± standard errors; those for other mutants correspond to a representative experiment. The lightly hatched boxes correspond to the activity of monomers and dimers (T_1+T_2) and the darkly hatched boxes to tetramers (homomeric tetramers T_4 and heteromeric T_4-P_{stpn} complexes). If homomeric T_4 tetramers are present in the same proportion to T_1 and T_2, as in the case of $AChE_T$ subunits alone, they represent a minor species and can be generally neglected. In mutants 16 to 18, the deletion of the N-terminal region is associated with mutations in the proline-rich segment, as indicated in parentheses. Note that a mutant reduced to the $RP_4LP_{10}RL$ motif (expressed as mutant Δ_N-R-P_{stp38}) induced the production and secretion of essentially the same amount of complex as the complete extracellular domain (P_{stp54} mutant). **C.** Correlation between the total secreted AChE activity and the proportion of tetramers in the medium. The numbers identify the mutants, as indicated in the left column of A. D. Correlation between the percentages of T_4 complexes in secreted and cellular AChE.

Fig. 6. Ratio of secreted to cellular activity of AChE forms. The secreted activity reflects the rate of secretion, whereas the cellular activity, 3-4 days after transfection, approximately corresponds a steady state value; therefore, these activities are not comparable, but their ratio may be considered as an index of the efficiency of secretion, for each AChE form. These ratios were arbitrarily normalized to 100 for AChE tetramers produced in co-transfection with the complete N-terminal domain of PRiMA, P_{stp54}. For monomers and dimers (T_1+T_2), the ratio remained approximately constant, in agreement with the fact that their structure did not change.

Fig. 7. Synthetic PRiMA peptides induce oligomerization of $AChE_T$ subunits *in vitro*. **A**. COS cells were transfected with $AChE_T$ and the synthetic peptides RP_4LP_{10} and $RP_4LP_{10}RL$ were added with fresh culture medium after 2 days. The cells were extracted 24 hours later and analysed by non denaturing electrophoresis, as well as the medium. **B**. The peptides were added at a concentration of 10^{-4} M to an homogenate of COS cells expressing only $AChE_T$ subunits, and incubated overnight at 37°C. In some samples, a synthetic t peptide (WAT) was added at 2 10^{-4} M, as indicated. The homogenate was then analyzed by non denaturing electrophoresis in the presence of detergents (see Methods). The peptides induced the formation of stable $AChE_T$ tetramers (indicated by arrows); this effect was reduced by addition of the t peptide (WAT).

Keywords: Acetylcholinesterase; PRiMA; PRAD; degradation; secretion; protein shipwrecking

Fig. 1


A
Sequence of mouse PRiMA
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N-term. domain
EPQKSCSKVTDSCQHICQCRPPPPLPPPPPPPPPRLLSAPAPNSTSCPAEDS
TM domain
WWSGLVIIVAVVCASLVFLTVLVII
C-ter. I
CYKAIKRKPLRKDENGTSVAEYPMSSSQSHKGVDVNAAVV
C-ter. II
CYKAIKRNQAI
Nflag-PRiMA
DYKDEPQKSCSKVTDSCQHICQCRPPPPLPPPPPPPPPRLLSAPAPNSTSCP....
B
Sequences of truncated PRiMA mutants
C-terminal truncations (Pstpn)
Pstp54
EPQKSCSKVTDSCQHICQCRPPPPLPPPPPPPPPRLLSAPAPNSTSCPAEDS
Pstp46
EPQKSCSKVTDSCQHICQCRPPPPLPPPPPPPPPRLLSAPAPNS
Pstp31
EPQKSCSKVTDSCQHICQCRPPPPLPPPPP
N-terminal deletions
ΔN-R-Pstp46
RPPPPLPPPPPPPPPRLLSAPAPNS
ΔN-E-Pstp46
E----------PPPPLPPPPPPPPPRLLSAPAPNS
Interaction motif
ΔN-R-Pstp38
RPPPPLPPPPPPPPRL
Mutations of leucines in the poly-proline stretches
P15
E----------PPPPPPPPPPPPPRLLSAPAPNS
P4LP5LP4
E----------PPPPLPPPPPLPPPPRLLSAPAPNS
P10LP4
E----------PPPPPPPPPPLPPPPRLLSAPAPNS
C
Sequence of the ColQ-derived N-terminal fragment
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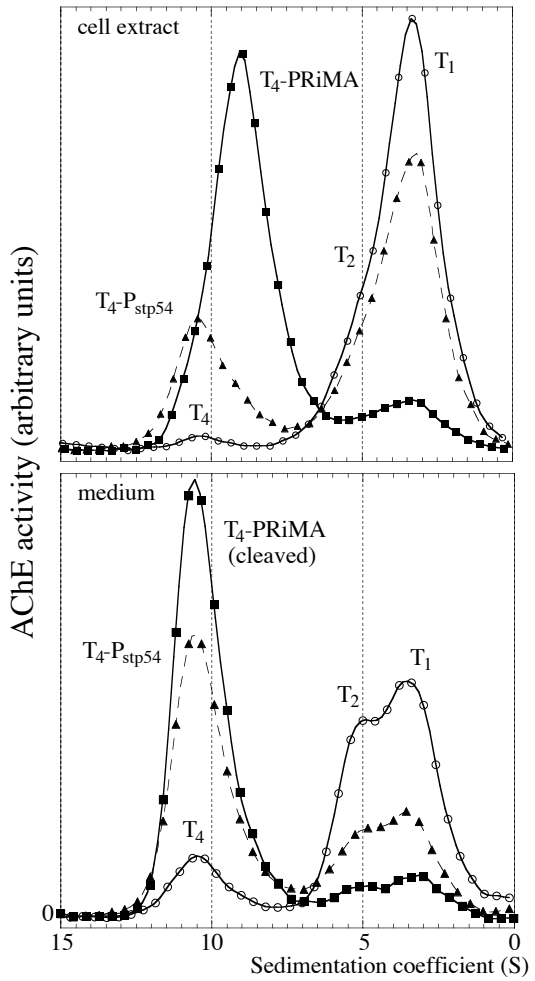
Fig. 2

A Sedimentation profiles



B Non-denaturing electrophoresis

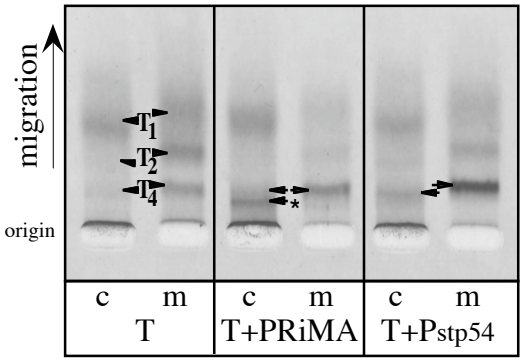


Fig. 3

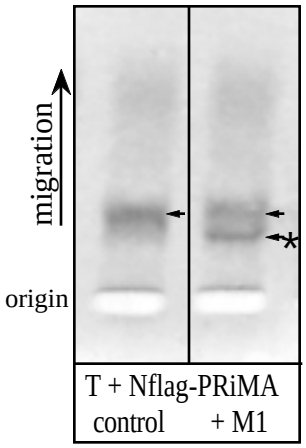
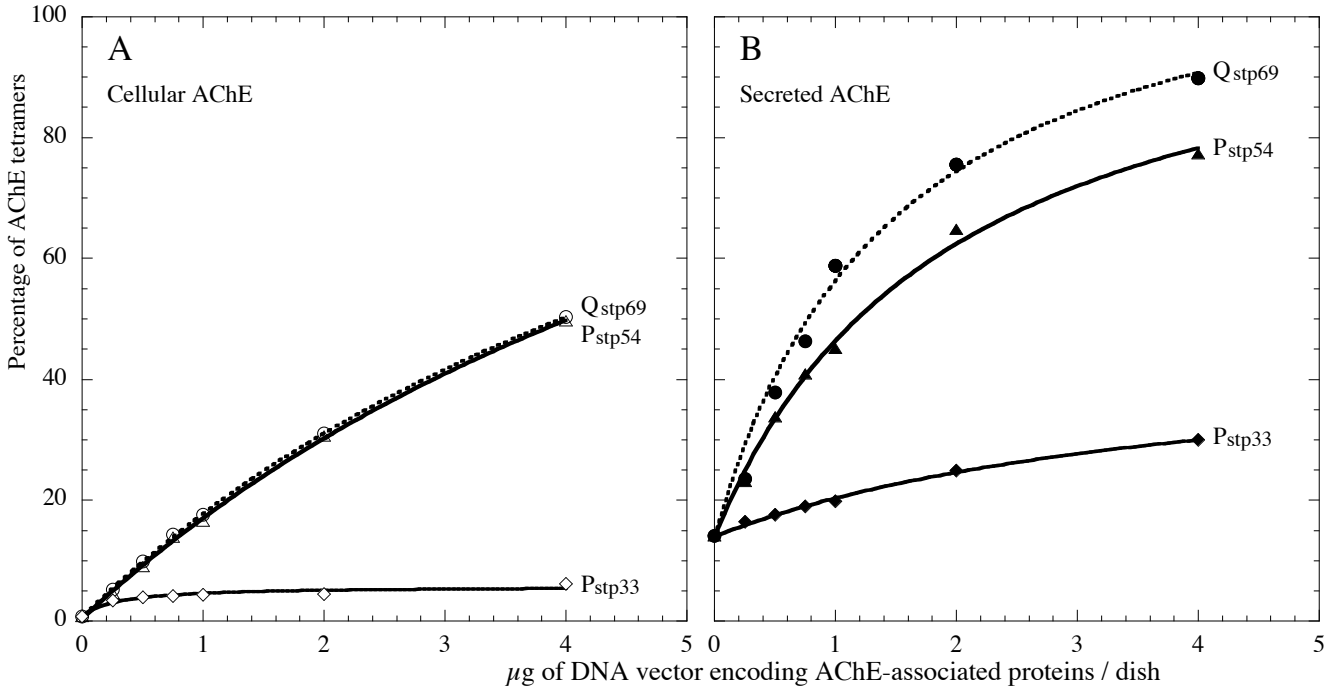
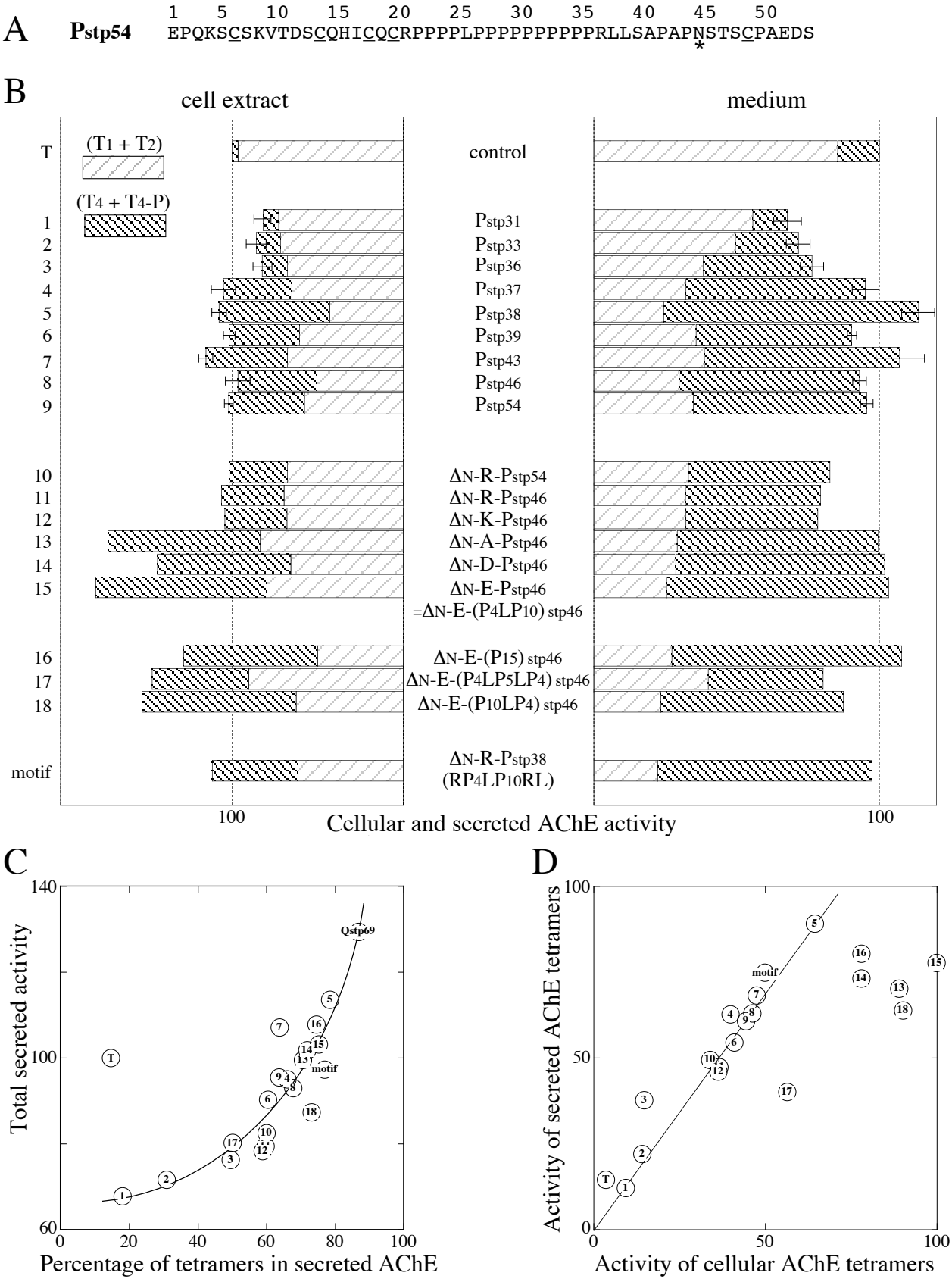


Fig. 4


A
Cellular AChE
B
Secreted AChE
Percentage of AChE tetramers
μg of DNA vector encoding AChE-associated proteins / dish
Qstp69
Pstp54
Pstp33
0
20
40
60
80
100
1
2
3
4
5


A
Pstp54
1 5 10 15 20 25 30 35 40 45 50
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*
B
cell extract
medium
T
(T1 + T2)
(T4 + T4-P)
control
1 Pstp31
2 Pstp33
3 Pstp36
4 Pstp37
5 Pstp38
6 Pstp39
7 Pstp43
8 Pstp46
9 Pstp54
10 ΔN-R-Pstp54
11 ΔN-R-Pstp46
12 ΔN-K-Pstp46
13 ΔN-A-Pstp46
14 ΔN-D-Pstp46
15 ΔN-E-Pstp46
=ΔN-E-(P4LP10) stp46
16 ΔN-E-(P15) stp46
17 ΔN-E-(P4LP5LP4) stp46
18 ΔN-E-(P10LP4) stp46
motif ΔN-R-Pstp38
(RP4LP10RL)
100
100
Cellular and secreted AChE activity
C
Total secreted activity
Percentage of tetramers in secreted AChE
Qstp69
motif
D
Activity of secreted AChE tetramers
Activity of cellular AChE tetramers
motif

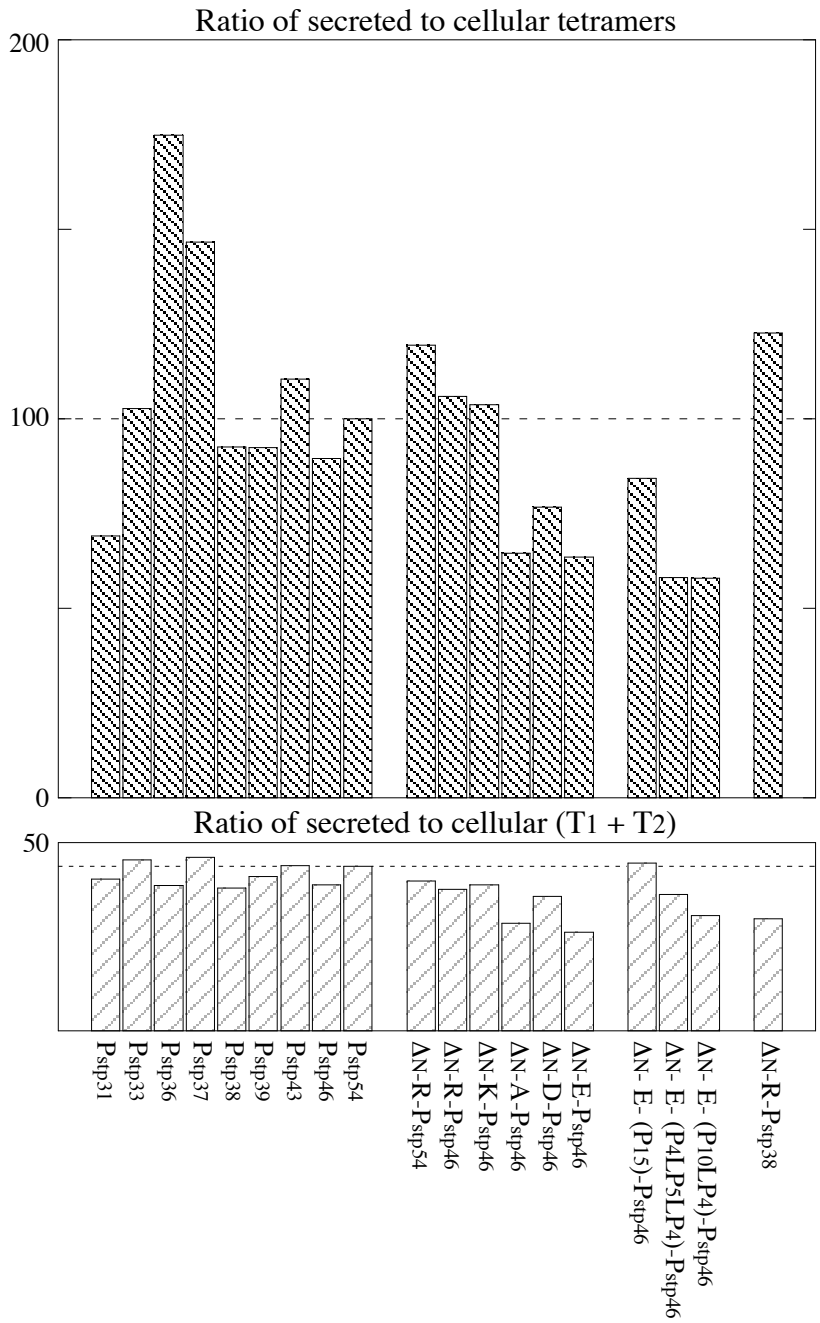
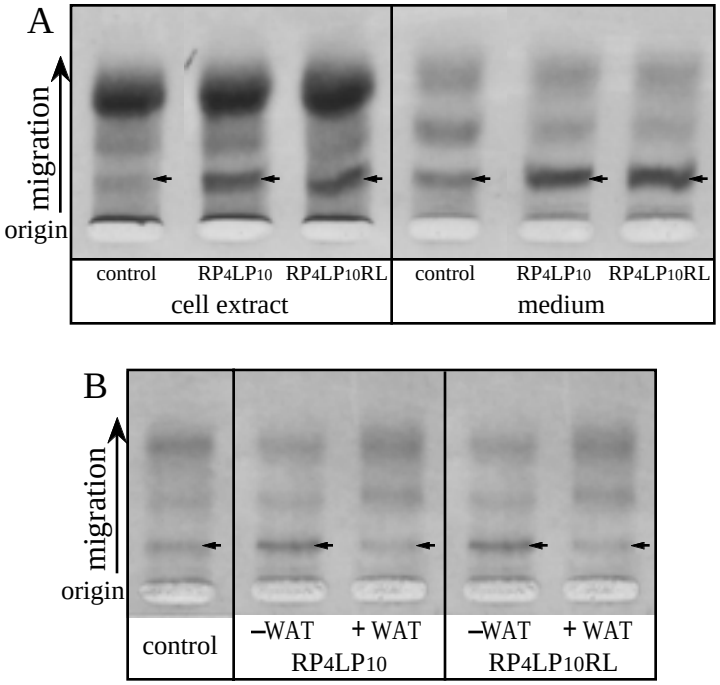

Ratio of secreted to cellular tetramers
200
100
0
Ratio of secreted to cellular (T1 + T2)
50
Pstp31
Pstp33
Pstp36
Pstp37
Pstp38
Pstp39
Pstp43
Pstp46
Pstp54
ΔN-R-Pstp54
ΔN-R-Pstp46
ΔN-K-Pstp46
ΔN-A-Pstp46
ΔN-D-Pstp46
ΔN-E-Pstp46
ΔN- E- (P15)-Pstp46
ΔN- E- (P4LP5LP4)-Pstp46
ΔN- E- (P10LP4)-Pstp46
ΔN-R-Pstp38

Fig. 7


A
migration
origin
control
RP4LP10
RP4LP10RL
control
RP4LP10
RP4LP10RL
cell extract
medium
B
migration
origin
control
–WAT
+WAT
–WAT
+WAT
RP4LP10
RP4LP10RL